CYTOMORPHOLOGICAL, PHYTOCHEMICAL AND MOLECULAR STUDIES OF GENUS *Physalis* L. FROM HIMACHAL PRADESH

SYNOPSIS

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1. Title: CYTOMORPHOLOGICAL, PHYTOCHEMICAL AND MOLECULAR STUDIES OF GENUS *Physalis* L. FROM HIMACHAL PRADESH

2. Introduction: Since ancient times, numerous ailments have been treated with the aid of plants and herbs in the preparation of medications. Herbal remedies use has grown during the last few years. As per WHO, most people on earth employ conventional techniques borrowed from plants for the therapeutic use of different health issues (Abo and Lawal, 2013; Agarwal, 1997). According to the literature, there are roughly 120 species of *Physalis* L. in the world, six of which are found in different parts of India (*P. alkekengi* L., *P. peruviana* L., *P. angulata* L., *P. longifolia* Nutt., *P. minima* L., and *P. ixocarpa* Brot. Ex. DC.). In discontinuous landscapes and crops throughout the tropics, including Asia, discrete species of the *Physalis* genus and their hybrids have become well-established weeds (Atta -ur- Rehman, 2005). Cytological analysis of the very first Indian species viz., *P. alkekengi* L. is 2n=2x=24 (Badr et al, 1997; Basey et al, 1992, Bastos et al, 2005). The species *P. angulata* L. was also cytologically well studied and reported with 2n=4x=48 (Bastos et al, 2008; Bir et al, 1978; Cassady and Suffness, 1980; Chapman, 1996). *Physalis* L. genus, has a chromosomal count of 2n=4x=48, while *P. longifolia* var. *longifolia* has a chromosome count of 2n=2x=24 (Chiang et al, 1992A). According to significant cytological research, the fourth Indian species *P. minima* L. has 2n=4x=48 and 2n=6x=72 chromosome numbers (Chaing et al, 1992B; Choi et al, 2006; Chopra, 1956; Choudhary et al, 2005). *P. peruviana* L., the fifth Indian species, was also investigated and found to have tetraploid and hexaploid chromosomal numbers of 2n=4x=48 and 2n=6x=72, respectively (Rao, 1979; Panda and Rao, 1983; Quiros, 1984). The cytological results demonstrate that distinct species of the Indian *Physalis* L. genus have different (2x, 4x, and 6x) ploidy levels. The goal of cytological research is to conserve medicinal plants' genetic diversity, particularly the germplasm of novel cytotypes, for future study. *Physalis* L. species include various lipids, minerals, carbohydrates, vitamins and phytosterols, secosteroids, withanolides, etc., with various health benefits. Different parts of the plant from the genus *Physalis* contain very important chemical components. These components are the different types of physalins, withanolides, saponins, fatty acids, flavonoids, steroids, and some very important vitamins, etc. (Garcia, 1975; Gengaihi et al, 2013; Glimore, 1977; Glotter et al, 1975; Glotter, 1991). Physalins

are under attention because of their anti-tumor and cytotoxic activities (Mericli, 2011; Milena et al, 2011; Mohammad et al, 2008). *Physalis* has many biological activities like anti-bacterial, anti-septic, abortifacient, molluscicidal, antiprotozoal, anticancerous, cytotoxic, and immunomodulatory activities. *Physalis* shows various major activities like immunomodulatory, anticancerous, antidiabetic, and hepatorenoprotective activity. The plant also shows minor activities like antimicrobial, antioxidant, molluscicidal, antispasmodic, and diuretic properties (Murali et al, 2013; Nanumala et al, 2012; Nathiya et al, 2012; Ng and Ser, 1988; Ngern et al, 2013; Nirmala et al, 2009). But the medicinal properties have not been used commercially for all six species. The enormous need for new cyst morphotypes to be kept on hand for future germplasm maintenance and evaluation is revealed by cytomorphological data. Nobody has been focusing on these crucial issues up until now. It is noted that additional chemotypes need to be identified based on reported phytochemical data for further allopathic and natural medication compositions. There is a strong desire and likelihood to discover new active ingredients and quantitative and qualitative study of unexplored *Physalis* species in India. These species displayed strong anti-inflammatory, immunomodulatory, anti-cancer, anti-asthmatic, anti-diabetic, and antibacterial properties, according to the findings published, etc. from different extracts and isolated phytoconstituents. Further, there is a huge need to identify new chemot ypes within these indigenous species with the help of new chromatographic techniques. According to the statistics, it is evident that six species have been extensively examined using various criteria, however, there is a need to do further *in-vitro*/*in-vivo* extensive bioactivities. Different studies and investigations showed that these plant species are mainly involved in immunomodulatory models and anticancerous agents. Hence there is a necessity to do extensive research work on the indigenous species of the genus *Physalis.*

Knowledge gap:

- There are only few studies available in literature related to gene or trait specific-markers used on genus *Physalis.*
- The genus *Physalis* still has undiscovered for *in-vitro* anti-cancerous properties in some species.
- Major phytochemical characterization analysis on diverse plant parts of distinct species of the genus *Physalis* needs to be further analyzed.
- Numerous cancerous such as hepatic and breast cancerous cell lines have not been yet tested with some of the selected plant species.

Objectives:

1) Collection and maintenance of plant species of genus *Physalis* L.

2) Detailed cytomorphological studies of the three selected species of genus *Physalis* L.

3) Phytochemical analysis of three selected species of genus *Physalis* L. using analytical techniques.

4) Extraction, isolation and characterization of active components in one of the selected species.

5) Molecular characterization of all the collected species through trait specific SSR markers.

6) To screen *in-vitro* antioxidant, anti-microbial and anti-cancerous bioactivities of selected species and isolated compounds.

3. Expected outcomes: Barely information is available about the molecular characterization of the biological diversity of selected genotypes. Few markers like SCoT, SCAR, and SSR markers were already studied but still, there is a great need to work with gene or trait specific-markers in some species of the genus *Physalis*. A number of cancerous cell lines were already tested with the important medicinal species of *Physalis*, many others such as breast cancer cell lines like MCF-7 and various adenocarcinoma cell lines like A549 are still pending testing. Major phytochemical characterization analysis still needs to be investigated further, on various plant parts of discrete species of the genus *Physalis*. Further, there is an urgent need to identify new chemotypes within these indigenous species with the help of new chromatographic techniques.

4. Review of Literature

4.1 Origin, morphology, and bioactivities: The Solanaceae (nightshade family), which includes the genus *Physalis*, is native to warm temperate and subtropical climates. This genus is distinguished by some little orange-colored fruits that resemble miniature tomatoes in terms of their size, shape, and structure, but are partially or completely enclosed in a large paper-thin husk derived from the calyx. Colombia is the world's leading producer and supplier to the European market, mainly to Germany and the Netherlands. Many *Physalis* species are also named ground cherries. A common name for *Physalis peruviana* is Cape gooseberry, also known as Rasbhari in India. It is native to South America but has been cultivated in several other parts of the world since the 18th Century. Basic studies on gooseberries have given us some preliminary proof that the phytochemical components of the fruit, presumably polyphenols or carotenoids, have anti-inflammatory and antioxidant qualities. (Wu et al, 2006; Pardo et al, 2008). It has also shown possible in vitro properties against diabetes and hypertension mechanisms (Pinto et al, 2009). When fully ripe, the fruit tastes delicious, rather like a cherry tomato used frequently to reduce pain (analgesic action). It has also shown analgesic and anti-inflammatory action in animals (Khan et al, 2009). In natural medicine, *Physalis* sp. has been widely used to treat conditions including asthma, dermatitis, diuretic, hepatitis, malaria, and rheumatism. It is also anticancer, antileukemic, antipyretic, antimycobacterial, and immuno-modulatory (Chiang et al, 1992a, 1992b; Pietro et al, 2000; Ismail and Alam, 2001; Soares et al, 2003; Patel et al, 2011). From the organic portions of *P. angulata*, physalins (such as Physalins A, B, D, and F) and glycosides (such as Myricetin-3-O-neohesperidoside) had been isolated (Chiang et al, 1992a, 1992b; Ismail and Alam, 2001; Soares et al, 2003). They have

anticancer effects on the cell lines HA 22T (hepatoma), KB-16 (epidermoid carcinoma of the nasopharynx), A498, ACHN, UO-31 (renal carcinoma), leukaemia, and lung adenocarcinoma. (Chiang et al, 1992a, 1992b; Wu et al, 2012; Ismail and Alam, 2001). In a study, physalin F was extracted from *Physalis angulata,* which appears to be a promising anti-cancerous agent by inducing cell apoptosis (Wu et al, 2012). Recently three new physagulidines A, B, and C were extracted from *P. angulata* and showed antiproliferative bio-activity against the DU145 prostate cancer cell line (Jin et al, 2012). Another recent study found that the withanolide from this plant, Physangulidine A, aids in the prostate cancer cell killing by the apoptosis induction process. Compound 4-hydroxywithanolide E, a *Physalis peruviana* extract, stops the growth of human lung cancer cells by inducing DNA damage, apoptosis, and G2/M arrest (Yen et al, 2010). Human head and neck squamous cell cancer (JMAR and MDA-1986), melanoma (B16F10 and SKMEL-28), and normal foetal fibroblasts (MRC-5) have all been shown to be very sensitive to cytotoxic withanolides from *P. longifolia* (Zhang et al, 2011). In a different study *Physalis* powder or extract effect with chromium studied in rats, shows an ameliorating effect on the health of rats (Hanna et al, 2012). The antidiabetic activities were shown by the *Physalis angulata* ethanolic root extract tested against the alloxan-induced rat models (Oladade et al, 2013). In a similar study, the alloxan-persuade diabetic rats were checked against the ethanolic extracts of *Physalis alkekengi,* the extracts showed positive results against diabetes. It has been concluded that these results may be due to the presence of a high concentration of citric acid, physalins, and vitamin C (Sanchooli et al, 2011). Another recent study on the diabetic activity of the leaf extracts of the *Physalis peruviana* in guinea pigs shows promising activity (Kasali et al, 2013). Potent antileishmanial activity of physalins B and F from *Physalis angulata* had been shown in in vitro and in vivo models of cutaneous leishmaniasis (Elisava et al, 2009). A recent in-Vivo study on the renoprotective activity of *Physalis peruviana* L. fruits shows very significant activity in improving renal injuries in rats (Ahmed et al, 2014).

4.2 Phytochemical analysis: Researchers worked on *Physalis angulata* and *P. lancifolia* for the isolation of chemical constituents. The results showed the isolation of five new physalins E, F, G, H, and I. The structural illucidation of physlain E and H established respectively as 5,6-dihydro-5α,7α-dihydroxy and 7β-hydroxy derivatives of physalin B (Row et al, 1978). Researchers developed a simple high-performance liquid chromatographic (HPLC) assay using the internal standard method for the simultaneous determination of five active ingredients from the calyces of *Physalis alkekengi* (L.). The results indicate that the HPLC method developed can easily be applied to the determination of five active ingredients in the calyces of *P. alkekengi* L. var. franchetti (mast.) (Baoli et al, 2012). *P. angulata* dry leaves were sequentially extracted with dichloromethane and 70% methanol. The results showed the isolation of four flavonoids through column chromatography from the n-butanol fraction. The spectroscopic data includes NMR and mass spectroscopy, the chemical structures were determined as quercitin (1), quercitin 3-O-methyl ether (2) isoquercitin (3) and Kaempferol 7-o-rhamnoside (4)

(Augustine and Ufuoma 2013). The peruviana L. extract was subjected to high performance liquid chromatography analysis for the identification of major flavonoids. The qualitative analysis revealed the presence of biologically active secondary metabolites such as phenols, flavonoids, glycosides, sterols, saponins, tannins, lactones and alkaloids in the fruit extract. The results of the present study indicated that many compounds of *P. peruviana* fruit are responsible for its pharmacological as well as beneficial effects (Sathyadevi and Subramaniam, 2014). The researchers had checked the volatile components in cape-gooseberry ripened fruits using headspace- solid phase micro extraction and analysed by gas chromatography-mass spectrometery. Total 133 volatile compounds were identified in fruit pulp; among them 1-hexanol (6.86%), eucalyptol (6.66%), ethyl butanoate (6.47%), ethyl octanoate (4.01%), ethyl decanoate (3.39%), 4-terpinol (3.27%), and 2-methyl-1-butanol (3.10%) were the major components in the sample extracts (Murat, 2014). In this study the researchers have developed a method based on high-performance liquid chromatography- photodiode array detection for the simultaneous determionation of three anti-proliferative withanolides (Withalongolide A, withaferin A and withalongolide B) present in the above ground biomass of the long-leaf groundcherry. Such an approach is applicable for the rapid detection and quantitative assessment of withanolides in *P. longifolia* (Cao et al, 2014).

Compound name	**Plant part**	**Bio- activities**
Physalins		
Physaperuvin A	Aerial parts (Xu et al, 2017/ Sang-Ngerm et al, 2016)	Anticancerous against ACHN- renal carcinoma cell line (Xu et al, 2017)
Physaperunolide A	Whole plant (Dong et al, 2019)	Cytotoxic against lung, breast, and liver cancer cell lines (Lan et al, 2009)
Peruvianolide B	Whole plant (Dong et al, 2019)	Anti-inflammatory effects on nitric oxide inhibition (Dong et al, 2019)
Peruvianolide C	Whole plant (Dong et al, 2019)	Anti-inflammatory effects on nitric oxide inhibition (Dong et al, 2019)
Peruvianolide D	Whole plant	Anti-inflammatory effects on nitric oxide inhibition (Dong et al, 2019)
Withanolides		
Phyperunolide E	Aerial parts (Lan et al, 2009)	Cytotoxic against Hep G2, Hep G3, Hep 3B, A549, MDA-MB-231, MCF-7

Phyperunolide F	Aerial parts (Lan et al, 2009)	Cytotoxic against Hep G2, Hep G3, Hep 3B, A549, MDA-MB-231, MCF-7
Phyperunolide A	Aerial parts (Lan et al, 2009)	Cytotoxic against Hep G2, Hep G3, Hep 3B, A549, MDA-MB-231, MCF-7
Withaperuvin L	Aerial parts (Fang et al, 2012)	Anti-cancerous against ACHN-renal carcinoma cell lines (Xu et al, 2017)
Withanolide FA	Aerial parts (Fang et al, 2010)	Strongly cytotoxic against ACHN-renal carcinoma cell lines (Xu et al, 2017)
4β-Hydroxy-withanolide E	Aerial part (Sakurai et al, 1976)	Anti-cancerous by modulation of splicing factors and histone modification (Lee et al, 2017), Cytotoxic against lung breast, liver and prostate cancer cell lines LNCaP and 22Rv1 (Xu et al, 2017), Cell cycle arrest and apoptosis down-regulation of Hsp90 client proteins histone modification (Park et al, 2016), Inhibition of tumor activity in carcinogenic progression (You et al, 2014), Inhibition of growth of human lung cancer cells through DNA- damage, apoptosis, and G2/M arrest (Yen et al, 2010); Cyclooxygenase-2; transcription of Inducible nitric oxide synthase (Park et al, 2019), Intracellular Anti-oxidant effect through inhibition by oxidative stress and Anti-inflammatory effect by suppression of COPD (Yang et al, 2020), Anti-oxidant enzyme by up-regulation of Nrf2 and Nrf2- downstream genes (Peng et al, 2016), and Anti-cancerous to tumor growth and the proliferation of colorectal cancer cells (Ye et al, 2019).
7β-Hydroxy-withanolide F	Aeroponic growth (Xu et al, 2017)	Cytotoxic against prostate cancer cell lines LNCaP and 22Rv1 (Xu et al, 2017)
Sucrose esters		
Peruviose A	Calyx and Fruits (Franco et al, 2014)	Anti-inflammatory in vitro inhibition of NO and prostaglandin E2 (Franco et al, 2014) and anti-diabetic activity against α-Amylase (Bernal et al, 2018)
Peruviose B	Calyx and Fruits (Franco et al, 2014)	Anti-inflammatory in vitro inhibition of NO and prostaglandin E2 (Franco et al, 2014) and anti-diabetic activity against α-Amylase (Bernal et al, 2018)

Peruviose C	Fruits (Bernal et al, 2018)	Anti-diabetic activity against α-Amylase (Bernal et al, 2018)
Peruviose D	Fruits (Bernal et al, 2018)	Anti-diabetic activity against α-Amylase (Bernal et al, 2018)
Peruviose E	Fruits (Bernal et al, 2018)	Anti-diabetic activity against α-Amylase (Bernal et al, 2018)
Peruviose F	Fruits (Bernal et al, 2018)	Anti-diabetic activity against α-Amylase (Bernal et al, 2018)

4.3 Ethno-botanical importance: The whole plant *P. angulata* is utilized as many ethno- botanicals in different countries. Fruits are edible and sweet-tart. Underground parts, like roots, are used for tea or infusion. In the Amazon, leaf concoction is used as a diuretic, and leaf juice is used for worms. A leaf infusion is used to treat postpartum infection in Peru. A fruit decoction is used in the Solomon Islands to treat infertility. In the West Indies and Jamaica, leaves are used to prevent miscarriages (Rengifo-Salgado et al, 2013; Sharma et al, 2015; Rathi et al, 2017). The fruit of *P. peruviana* is considered an edible, raw, or cooked material. Fruits are also rich in A, B, and C-complex vitamins. These are excellent for making jams and preservatives for pies and cakes (Singh et al, 2019). In India, it is used by the Tamilian and Muthuvan tribes for the treatment of jaundice. In the Western Ghats, India, leaves and dried seeds are used to treat jaundice and glaucoma (Anonymous 1). The Puebloan people called the fruits of *P. longifolia charka* and *shumacharoka* and ate them fresh or cooked (Kindscher et al, 2012).

4.5 Genetic diversity: The evaluation of genetic diversity is necessary to identify genotypic groups and to utilize the genetic resources. Genetic diversity can serve as a possible foundation to recognize the genes leading to the improvement of cultivars (Govindaraj et al, 2015). The most used marker is RAPD, as it is simple and has relatively low cost, but also has some limitations of non-reproducibility. Other markers, such as SSR and AFLP are preferred for analysis due to high reproducibility, polymorphic, multi-allelic, and co-dominant (Garcia et al, 2004). SSR marker is time-consuming and also costly, while microsatellite markers are easily transferable across taxonomic margins. This feature is very important for developing a good DNA marker (Vieira et al, 2016).

Since the 1980s, many scientists have tried to create DNA markers along with linkage maps for tomatoes, potatoes, pepper, and eggplant. Analysis of the characteristics provides a strong foundation for breeding programs because the majority of essential agronomical traits, such as harvest quality, yield, and resistance to biotic and abiotic challenges, are caused by the presence of quantitative trait loci (QTL). Tomato is a prototype species for solanaceous crops, as most of the information on molecular breeding is available in this species (Fukuoka, 2016). SSR markers are polymorphic, multi-

allelic among closely related breeding species, and evenly distributed throughout the whole genome can be used for selectable marker development for traits of interest (Fukuoka, 2016).

The paucity of molecular markers in *Physalis* makes it difficult to conduct in-depth genetic and phylogenetic research, which hinders breeding development even further (Wei et al, 2012). Phylogenetic analysis for the prediction of self-incompatibility alleles in *P. cinerascens* and *P. crassifolia* was done by Richman and Kohn, (1999). Garcia-Arias et al, (2018a) discovered 36,142 instructive SNP markers in *P. peruviana*, based on the transcriptome analysis of 18 characters relating to fruit quality, fruit yield, and fruit size and form from 100 accessions. Morphology and genetic diversity of sixteen accessions of *P. ixocarpa* were studied by Khan et al, (2019) and found that genetic diversity deviated from morphology due to the influence of environmental factors on qualitative and quantitative characters. Wei et al, (2012) derived 25 SSR markers from *P. peruviana* along with 97 tomato markers to study the genetic diversity. Between them, they come across 3356 single-copy orthologous genes. Comparing the leaf transcriptome of the species with tomato, potato and from assembled ESTs formulated 5,971 new SSR markers. Later, 47 accessions of this species along with 13 accessions of related taxa were analyzed for their genetic diversity and population structure based on InDels and SNPs derived from COSII and IRG markers (Garzón-Martínez et al, 2015). Usaizan et al, (2018) collected 19 accessions of *P. minima* from eleven states of Peninsular Malaysia to analyze diversity based on morphological characteristics. 85 Colombian accessions of *P. peruviana* were analyzed using 15 SSR markers and 60,663 SNPs associated with the Cape gooseberry resistance for vascular wilt caused by *Fusarium oxysporum* were identified by (Chacon et al, 2016; Osorio-Guarín et al, 2016) respectively. A mutational approach causing genetic variations is also used in *Physalis* for the enhancement of fruit quality (Trevisani et al, 2017).

Genetic differences among accessions from different origins, for yield and fruit quality characters, can be exploited for Cape gooseberry breeding. Leiva-Brondo et al, (2001) evaluated vegetative characters, fruit yield, weight and shape, and L-ascorbic acid content in three hybrids and their parents of Cape gooseberry grown outwards and inside the glasshouse. Maximum yields were procured with hybrids, specially grown in a glasshouse.

Kumar et al, (2018) compared genotypes from Srinagar and Bihar using RAPD primers. They suggested that genotypes from a former area are better and can be used as a diverse parent in hybridization programs or as direct selection. Previously, Kumar et al, (2017) recorded the highest values of phenotypic and genetic coefficient of variation for fruit, fruit diameter, fruit weight, and flowers per branch. Therefore, choosing superior parents with these high qualities that can be employed for hybridization is crucial for crop improvement. Garcia-Arias et al, (2018b) produced double haploids from axillary buds from the haploid genotypes of *P. peruviana* by the treatment of colchicine (5-10mM). The mixoploids formed are fertile and have darker green larger leaves, flowers, and fruits. Previously, Caro-Melgarejo et al, (2012) used 100Gy of gamma radiation to induce genetic variability in seedlings associated with better phenotypic characteristics. Ramírez-Godina et al, (2013) evaluated auto-

tetraploid and diploid populations of *P. ixocarpa* from Mexico for yield and fruit quality. Genome duplication has a positive effect on nutritional quality, and there is a change in the shape of the fruits. The grafting technique has also been used in Solanaceae crops to improve fruit quality, manage soil-borne diseases, and develop abiotic stress resistance (King et al, 2010).

5. Technical Programme

SSSSS5.1. Objective 1. Collection and maintenance of plant species of genus *Physalis* L.

5.1.1. Experiment 1: Collection of seeds

5.1.1.1. Location: The seeds of *Physalis* L. genus were collected from Department of Botany, Eternal University, Baru Sahib, H.P.

5.1.1.2. Methodology: The selection of best germplasm was based on the results of previous research studies. Germplasm with highest phytochemical content for each species will be selected.

5.1.1.3. Observation: The finest germplasm of *P. angulata* L. was discovered in the district of Hamirpur, Himachal Pradesh. Whereas, the best germplasm of *P. peruviana* L. and *P. longifolia* Nutt. originated from the Kalka area in Haryana and the Sirmour region in Himachal Pradesh, respectively.

5.1.1.4. Statistical Analysis: Nil.

5.1.2. Experiment 2: Maintenance of plant species

5.1.2.1. Location: Plants will be maintained at polyhouse of Eternal University, Baru Sahib, H.P.

5.1.2.2. Methodology: The seeds of selected germplasm were planted inside the polyhouse of Eternal University and maintained under local environment conditions for the purpose of conducting research.

5.1.2.3. Observation: Understand the plants natural habitat, including soil type, sunlight requirements, water needs and climate conditions. Note the plant's ecological niche and growing preferences.

5.1.2.4. Statistical Analysis: Nil.

5.2. Objective 2. Detailed cytomorphological studies of the three selected species of genus *Physalis* L.

5.2.1. Experiment 1: Morphological Analysis

5.2.1.1. Location: Plants will be studied from Herbal-cum-botanical garden and polyhouse of Eternal University, Baru Sahib, H.P.

5.2.1.2. Methodology: Characters such as plant height, the shape of leaves, flowers, fruit, seed colour, etc. will be recorded from fresh plant material in the field. The plants' identities will be validated by a taxonomy expert using genuine specimens kept in the herbarium of the department of taxonomy, at CSIR- Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, Himachal Pradesh India, and by referencing various floras of the region of North-Western India (Hooker, 1885; Aswal and Mehrotra, 1994). The authorized National Herbarium will receive all specimens.

5.2.1.3. Observation: The extent of morphological variability in the germplasm of collected accessions will be studied.

5.2.1.4. Statistical Analysis: Mean± Standard deviation.

5.2.2. Experiment 2: Cytological analysis

5.2.2.1. Location: The experiment will be performed in the Department of Botany, Eternal University, Baru Sahib, H.P.

5.2.2.2. Methodology: Flower buds of the appropriate size will be collected from plants, fixed in Carnoy's fixative for 24 hours, and then preserved in 70% alcohol for cytological investigation. Photomicrographs of transient preparations were taken (Iqbal and Datta, 2007).

5.2.2.3. Observation: By examining PMCs, the number of chromosomes will be counted.

5.2.2.4. Statistical Analysis: None.

5.3. Objective 3. Phytochemical analysis of three selected species of genus *Physalis* L. using analytical techniques.

5.3.1. Experiment 1: Phytochemical analysis

5.3.1.1. Location: Some of the preliminary experiments and HPLC analysis will be conducted in the Department of Botany, Eternal University, Baru Sahib, H.P. and the remaining major phytochemical experiments such as GC-MS and LC-MS analysis will be outsourced (CSIR-IHBT-Palampur).

5.3.1.2. Methodology: The preliminary and phytochemical analysis of methanolic-water (80:20) extracts of leaves and fruits from all three species of *Physalis* L. will be performed utilizing different analytical techniques such as HPLC, LCMS, GCMS (Slight modifications may take place with the procedures of given references).

A- Preliminary analysis: For this, above mentioned extracts with different concentrations will be used for a comparative study of total phenolic content, total flavonoid content, and total antioxidant content which results in the selection of elite germplasm.

B- Phytochemical analysis:

1) RP-HPLC-PDA Analysis: Part-based HPLC analysis of some vitamins, polyphenols, and isolated compounds will be performed by following the collection of plant materials, preparation of the sample and standard solutions, HPLC instrumentations and Chromatographic conditions, following method validation through calibration curve (Sharma, 2013).

2) GC-MS Analysis: GC-MS analysis will be carried out using simple derivatization methods (Ramakrishna Pillai et al, 2022).

3) LC-MS Analysis: It includes extraction procedures, biochemical investigation, and components identification/ characterization based on the charge-mass ratio (Zhang et al, 2018).

5.3.1.3. Observation: Vitamins like thiamin, riboflavin, niacin, and ascorbic acid, etc. whereas polyphenols such as rutin, syringic acid, epicatechin, gallic acid, caffeic acid, etc. and isolated Physalin

compounds will be checked quantitatively and qualitatively through HPLC, volatile components/oil extraction with GC-MS and compound identification will be observed with LC-MS analysis.

5.3.1.4. Statistical Analysis: Mean± Standard deviation.

5.4. Objective 4. Extraction, isolation and characterization of active components in one of the selected species.

5.4.1. Experiment 1: Preparation of Plant Extracts:

5.4.1.1. Location: Extraction will be performed at Department of Chemistry, CSIR-IHBT, Palampur, H.P.

5.4.1.2. Methodology: The air-dried materials of the chosen *Physalis* L. germplasm will be extracted using methanol and water (80:20) for three days at room temperature. After that, the extracts will be filtered. At 40 degrees, the filtrates will be gathered and concentrated at reduced pressure.

5.4.1.3. Observation: Different Solvents extraction will be analysed.

5.4.1.4. Statistical Analysis: Nil.

5.4.2. Experiment 2: Purification of Novel Phyto-constituents

5.4.2.1. Location: Purification will be performed at Department of Chemistry, CSIR-IHBT, Palampur, H.P.

5.4.2.2. Methodology: The *Physalis* L. extracts will then be split by N-hexane, ethyl acetate, butanol, and water after being disintegrated in water to isolate purified compounds.

5.4.2.3. Observation: The quantity of chemicals used and how much retention time is used by each isolated pure compound will be observed.

5.4.2.4. Statistical Analysis: Mean± Standard deviation.

5.4.3. Experiment 3: Characterization of Novel Compounds

5.4.3.1. Location: Characterization will be performed at the Department of Chemistry, CSIR-IHBT, Palampur, H.P.

5.4.3.2. Methodology: Various analytical techniques such as TLC, column chromatography, HPLC, LCMS, FTIR, NMR (^{1}HNMR AND ^{13}CNMR), etc. shall be developed for the characterization of pure phytoconstituents.

5.4.3.3. Observation: Identification, quantification, qualification, and structure of isolated pure compounds will be studied through above mentioned analytical techniques.

5.4.3.4. Statistical Analysis: Standard deviation.

5.5. Objective 5. Molecular characterization of the collected species through trait specific SSR markers.

5.5.1. Experiment 1: Isolation of DNA and PCR application

5.5.1.1. Location: Isolation will be done at Department of Genetics and Plant Breeding, Eternal University, Baru Sahib, H.P. PCR will be performed at Department of Genetics and Plant Breeding, CCS University, Meerut, U.P.

5.5.1.2. Methodology: Standardized crushing methodology using CTAB buffer will be performed. Different trait-specific markers will be used to characterize all the selected species of the genus *Physalis*. Details of different trait-specific markers including their sequence (Forward and backward) and their annealing temperature is given in the table below. PCR protocol will be followed as given by Simbaqueba et al, 2011.

Marker name	Type of marker	FORWARD (5'-3')	REVERSE (3'-5')	Annealing temparature
pSSR2	SSR	CATTGGGTTTCGCATCCAT	AGACAAGCCTAGGGGAAAGG	54°C
pSSR15	SSR	GCTTGTTGATCAGCTTTCTTTG	TGGATCATAACCTTGCTAATGC	52°C

5.5.1.3. Observation: Banding patterns of PCR will be analysed through a gel documentation instrument.

5.5.1.4. Statistical Analysis: Nil.

5.6. Objective 6. To screen *in-vitro* antioxidant, anti-microbial and anti-cancerous bioactivities of selected species and isolated compounds.

5.6.1. Experiment 1: Evaluation of *in-vitro* bioactivities

5.6.1.1. Location: Antioxidant and antimicrobial activities will be performed at Department of Botany, Eternal University, Baru Sahib, H.P., whereas anti-cancerous activity will be outsourced (IIIM-Jammu).

5.6.1.2. Methodology:

1) Antioxidant Activity: The antioxidant activity will be performed with methanolic-water (80:20) plant extracts of all the selected species. Based on various in-vitro assays (Enzymatic and Non-Enzymatic), it includes Radical Scavenging Assays, Metal Chelation Assays, Hydrogen, and Electron Donating Assays. Three different anti-oxidant assays will be performed to check anti-oxidant activity.

I. DPPH: 2-2-Dipheny l-picryl-hydrazyl Assay by Blois, (1958).
II. ABTS: 2-2'-Azino-bis (3- ethyl benzothiazoline-6-sulphonic acid) diazonium salt by Sun et al, (2014).
III. FRAP: Ferric Reducing Antioxidant Power Assay by Vijayalakshmi, (2016).

2) Anti-microbial activity: Different methanolic-water (80:20) extracts of various plant parts of selected species will be screened for different pathogenic bacterial and fungal strains by disc diffusion assay as described by Perez and co-workers with certain modifications. Nutrient agar will be used as the bacteriological medium (Perez et al, 1990).

3) Anti-cancerous activity: MTT Assay method will be carried out with methanolic-water (80:20) extract of leaves as described previously (Mossman, 1983). The cancer cell lines can be used to investigate the anticancer potential of selected sp. of genus *Physalis* L. We will select a few of them to do further study.

1. Breast cancer cell lines: MCF-7
2. Hepatic carcinoma Cell lines: Hep G2

5.6.1.3. Observation: Through the absorbance spectrum, results will be concluded that which plant part is more biologically active.

5.6.1.4. Statistical Analysis: ANOVA for mean ± Standard deviation analysis.

6. Collaboration: IHBT (CSIR) Palampur (H.P.) and CCS University, Meerut (U.P.).

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